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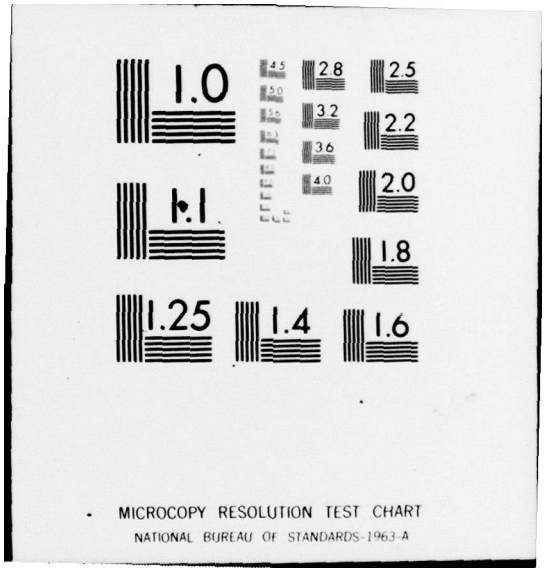
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Immunological Studies on Heroin Addiction

Final Report

Chi-Tan Liu, Ph.D. and Frank L. Adler, Ph. D.

October 1975

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Hemagglutination-inhibition (HI) procedures for the detection of morphine, methadone and barbiturates have been devised, developed and validated to the point of their adoption by diagnostic laboratories, and the necessary reagents have become available through commercial sources. The HI test for the detection of the major cocaine metabolites suffers from interference by unidentified substances found in some human urine specimens. After appropriate adjustment for this interference the test detects cocaine metabolites for about 4 days after

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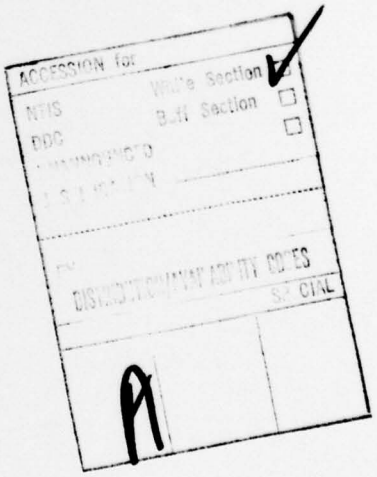
last use of the drug. HI tests for detection of amphetamine and LSD have yet to be completely developed. The application of serological methods for the study of pharmacokinetics has been critically examined and the results have been submitted for publication. Finally, the generation of specific antibodies in response to morphine administration has been verified, but the biological significance of this phenomenon remains in question.



Introduction

The general purpose of studies that were supported by this contract has been that of developing and of validating specific, rapid, sensitive inexpensive and reporducible test procedures for the detection of commonly abused drugs and their metabolites in biological fluids. Concurrently with this work, but supported by funds from other sources, additional studies have been conducted to probe certain immunological aspects of drug addiction. Included in the latter studies have been immunogenetic investigations into responsiveness to specific drugs, studies on actively acquired hypersensitivity to drugs after the repeated administration of the drug alone or in the form of drug-protein conjugates, and an examination of the possible role of circulating antibodies that react with the drug in the genesis of drug tolerance. Still other studies have concerned themselves with the technology of solid phase immunoabsorbants and their use in attempts to concentrate and purify drug or metabolites from urine.

As far as fiscal and purely administrative aspects were concerned, no major effort was required to distinguish between the practical and applied aspects of the work on the one hand, and the more theoretical or basic aspects on the other. Contract funds were applied to the former; grant and other funds to the latter. It proved far more difficult, however, to separate the various phases of the work intellectually or conceptually and, for this reason, we have considered it only reasonable to acknowledge support from more than one source in those of our publication that bridged the gap between basic and applied aspects of the work.

In presenting this final report on our contract-supported work, we shall not burden the reader with repetitions of what has been published or reported in detail in the several annual reports. More detail will be provided for work that is in preparation for publication. We shall also briefly summarize some of the approaches that proved to be disappointing or which have not yet yielded useful information. For the sake of convenience, the reported results will be organized according to the particular drug and group of drugs studied. Finally, it will be recalled that an additional report was filed in July 1975 as part of our unsuccessful re-application, and that contract funds supported this work only for three months beyond the report date.

Results

A. Morphine (heroin)

While the principle of immunization with conjugates of carboxymethylmorphine and proteins had been developed and reported by others for the purpose of obtaining antibodies that react with morphine, we had to invest considerable time and effort to reproduce this work and finally succeeded only after modifying the published procedures. When the contract began

to support our work, we had passed through this phase and had begun to develop the hemagglutination-inhibition (HI) test. The annual reports submitted during the period 1972-1974 and our several relevant publications describe procedures and results obtained both under controlled laboratory and under field conditions. We have had occasion to compare the hemagglutination-inhibition procedure which we had developed with alternate procedures, such as the radioimmunoassay and the enzyme activation procedure ("EMIT"). The published results show that HI is as sensitive as the radioimmunoassay (RIA), that is, is more sensitive than "EMIT", and that the specificity of the HI test is similar to that of the alternate procedure. Costs of HI are, of course, well below those of the other tests.

It may be considered a measure of the success of the HI test for morphine that this test has been further developed and is being distributed by commercial sources, that it is in use in several of the larger commercial laboratories, and - finally - that it has been singled out by a committee of WHO as the test procdure most applicable for use in the laboratories of the developing countries.

Still unfinished studies on the use of solid phase immunoabsorbants for the retrieval of morphine or other heroin metabolites have been discussed in our report of June 1974. We have no additional data of substance.

Observations on the recurrent sporadic excretion of "morphine equivalents" are being prepared for publication. While we have failed, thus far, to obtain positive identification of the immunologically reactive material to have adequate data to rule out morphine itself or any one of the known major metabolites. It is equally unlikely that the substance is one or the other of the endogenous peptides with morphine-like activity that have recently received so much attention. None of the peptides tested by us is bound by the antisera that we used in the detection of morphine.

The completed studies on the induction of serum antibodies that are reactive with morphine by repeated administration of morphine pellets under closely defined conditions, our evidence for the generation of specific immunological unresponsiveness (tolerance in the immunological sense) by morphine injections, and the suggestive evidence for a cellular immune response to morphine are all contained in two papers that are to appear in the International Archives of Allergy and Applied Immunology (July 1976). Preprints were submitted as part of our July 1975 report.

B. Cocaine and Metabolites

The development of a hemagglutination-inhibition (HI) test for the detection of cocaine and cocaine metabolites was begun in 1972 and some of the progress in these attempts has been cited in several of the annual reports. Since the material has yet to be published, we shall present this subject in greater detail.

The immunization of rabbits with ecgonine-bovine serum albumin (Ec-BSA) conjugates yielded, in the initial series of eight rabbits, highly suitable antibodies in repeated bleedings from one of the eight animals. Serum from this rabbit was used to develop the HI test in parallel with a radioimmunoassay and to apply the developed test to a large series of biological specimens. While the results of these studies were satisfying, we were hesitant to publish our findings because we failed to elicit antibody production against Ec-BSA in an additional twelve rabbits and also failed to obtain antibody formation in response to benzoylecgonine in an additional group of rabbits. Thus, our experience indicated that antibody formation could not be induced at will and that our experiment would come to an end with the exhaustion of sera from the one rabbit. The recent introduction of two alternate serological tests for cocaine abuse by two commercial sources (Syva and Technam) and information obtained from the suppliers of reagents both confirm our observations and, at the same time, provide for a continuing source of antibodies. We have learned that in the hands of others approximately 10 per cent of immunized animals (rabbits, goats or sheep) produce measurable antibodies in response to injection with conjugates of benzoylecgonine and protein. This, of course, is not significantly differnt from our finding and suggests that the material is either poorly antigenic or that genetic factors play an important role in this specific response.

It should be stressed that the serological test to be described is more properly considered as a test for cocaine metabolites (ecgonine and benzoylecgonine) than for cocaine proper. This is illustrated in the table where it will be noted that for ecgonine and benzoylecgonine are both much more effective inhibitors of the reaction than is cocaine. It will also be noted from the table that within the context of our test there exists no significant difference in the specificity of our rabbit serum (Rab 768) and three sheep antisera against benzoylecgonine obtained from Technam (courtesy of Dr. George H. Sherr).

Specificity of antisera against ecgonine and benzoylecgonine

Antiserum	Relative inhibitory activity		
	Ecgo-nine	Benzoyl-ecgonine	Cocaine
Rab 768 (anti-ecgonine)	1	0.25-0.5	100
Sheep 71-195E (anti-benzoyl-ecgonine)	1	0.5	1000
Sheep 30-11-A	1	0.25	1000
Sheep 30-11-B	1	0.12-0.25	1000

Using erythrocytes coated with conjugate of protein and ecgoinine and the antisera just described, we have tested the specificity of the reaction

with respect to drugs that are either chemically related or that might be found in biological specimens together with evidence of cocaine abuse. Such structurally similar compounds as atropine, scopolamine and various synthetic local anesthetics had no significant cross reaction. Similarly, a number of drugs that resemble cocaine in their pharmacological activities, such as amphetamines, epinephrine, etc., failed to interfere. Morphine, methadone and quinine also were found to be non-interfering. Using either our rabbit serum or the commercially available sheep sera, we found the sensitivity of the test to be such that 0.4-0.8 ng/ml of ecgonine (or ecgonine equivalents) could be detected and measured. It should be noted that the EMIT test requires minimal concentrations of 0.5-10/ug/ml, a 1000-fold difference in sensitivity.

In returning to the fact illustrated in the table that cocaine itself reacts poorly or not at all in the test, it seems important to call attention to the fact that aqueous solutions of cocaine deteriorate and thereby gain in serological reactivity in our test. The increase in serological activity can be correlated with an increase in chromatographically detectable benzoylecgonine and, eventually, in the presence of increasing amounts of ecgonine. It appears that solutions of ecgonine are the most stable and most desirable standards for the test.

In our preliminary attempts to apply the test to biological specimens, we tested urine specimens from three monkeys which had been injected with 1 mg/kg cocaine-HCL. These specimens had been made available to us by COL Demaree of WRAIR to whom we are much indebted. It was found that cocaine metabolites were rapidly detectable for 72 hours after adminstration of this dose. In an additional monkey whom we injected with a single intramuscular dose of cocaine (1 mg/kg cocaine-HCL), we found detectable excretion of ecgonine equivalents for a period of 4-5 days. A group of excreted ecgonine equivalents in detectable amounts for a similar period of time. From other studies, we were very much aware that animal models might not be relevant to human patterns. For example, we had found that methadone equivalents were quite rapidly eliminated in mice or monkeys given a single dose of methadone while excretion in humans volunteer, we have found that after a single small IV dose of 20 mg cocaine-HCL ecgonine equivalents were detectable in urine for 3-4 days and in serum for 2 days.

The observations just described pertain to the detection of ecgonine in amounts that are significantly above "background" where "background" is defined as apparent ecgonine concentrations found in normal specimens. We have found no significant "background" in the urine specimens of mice and of monkeys. This does not hold true for humans, however. In tests or urine specimens from 150 individuals who had received no cocaine it was found that approximately 25 per cent yielded false positive reactions for the presence of ecgonine. While most of these specimen appeared to contain 50 to 100 ng ecgonine equivalents/ml, a rare sample would contain an apparent 240 ng/ml. The reactive material interferes in an apparently

specific manner in the test for ecgonine alone and it is found rather consistently in urine specimens from some individuals and not in specimens from others. Its origin and nature are currently under investigation.

The practical significance of this finding is the following: (1) The sensitivity of the test for ecgonine must be reduced to the 250 ng/ml or higher level when the test is applied to a collection of random human specimens. (2) At this level of sensitivity, the test will still detect use of cocaine for at least 3-4 days after a small dose. (3) As discussed on previous occasions, the serological tests for drugs, at whatever level of sensitivity they may be done, are properly used only for the purpose of exclusion. Positive tests will always require confirmation by chemical analysis.

C. Other Drugs

No substantial progress has been made during the last three months of the contract in studies on other drugs and the annual report submitted in July 1975 represents the most up-to-date information from our laboratory.

To summarize such work briefly: We have satisfied ourselves that our test for methadone detects use of the drug for a period of at least one week beyond the last exposure even though the test does not detect the major cyclic derivatives of the drug. Attempts to prepare antibodies reactive with such cyclic metabolites are therefore not necessary for diagnotic purposes, but we are still attempting to make such antisera for research purposes. We have reported that antibodies reactive with amphetamines have been prepared, that their reaction with the drug can be demonstrated in a radioimmunoassay, but that we have been unable to develop an effective HI test in this system. Data descriptive of our HI test for the detection of barbiturates were submitted with our 1974 annual report. For the latest data on serological testing by HI for LSD we refer to the report of July 1975, in which we mentioned certain technical difficulties in the application of the test to urine specimens and pointed out that the amounts of this group of drugs that provide effective doses are so small that even the highly sensitive serological tests may not be capable of detecting their use beyond the initial 24-48 hour period.

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Louis De Cato, Jr., Ph.D.	Principal Professional Assistant
Jiri T. Beranek, M.D.	Professional Assistant

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